PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 48/00 | A2

(11) International Publication Number: WO 99/42137

(43) International Publication Date: 26 August 1999 (26.08.99)

(21) International Application Number: PCT/IB99/00294

(22) International Filing Date: 18 February 1999 (18.02.99)

(30) Priority Data:
60/075,267 19 February 1998 (19.02.98) US

(71)(72) Applicant and Inventor: BROMLEY, Peter [GB/CH]; 37, Crêts-de-la-Neige, CH-1234 Vessy (CH).

(74) Agent: ROLAND, André; 38, rue du Petit-Chêne, Case postale 1259, CH-1001 Lausanne (CH).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: STRESS PROMOTER CONTROL OF THERAPEUTIC GENES IN GENE THERAPY: COMPOSITIONS AND METHODS

(57) Abstract

The present invention relates to methods and compositions for the treatment of diseases by gene therapy. More particularly, it relates to the control of therapeutic gene expression by heat shock or stress promoters, the use of mixtures of genes for specific treatments, and the use of optimal induction protocols and systems.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

STRESS PROMOTER CONTROL OF THERAPEUTIC GENES IN GENE THERAPY : COMPOSITIONS AND METHODS

Field of the Invention

This invention relates to methods and compositions for the treatment of diseases by gene therapy. More particularly, this invention relates to the control of therapeutic gene expression by heat shock or stress promoters, the use of mixtures of genes for specific treatments, and the use of optimal induction protocols and systems .

Background of the Invention

Gene therapy is a rapidly evolving field of medical treatment that is finding applications in a wide variety of diseases, including genetically inherited diseases, cancers and infectious diseases. The basic difference in approach from the use of pharmaceuticals is that instead of the protein product itself being employed, such as with interferons, interleukins, growth hormones, blood clotting factors, insulin, vaccines etc., the DNA encoding such proteins is employed.

Two basic approaches to gene therapy have been tried to date, and these are the *ex vivo* approach, in which cells are removed from a patient, genetically modified by DNA transfections, and subsequently re-introduced into the patient, and *in vivo* approaches whereby the therapeutic DNA constructs are introduced into the patient's body as naked DNA or through the use of viral and non-viral vectors.

More and more genes of human origin with precise linkages to specific human diseases are being identified and isolated, and this through the many human genome projects ongoing today. Accordingly, more potentially useful therapeutic genes are becoming available for use in gene therapy. The classes of genes that are of interest include tumor suppresser genes such as p53 (Takahashi et al. Cancer Res. **52**, 2340-2343, 1992. Wild-type but not mutant p53 suppresses the growth of human lung cancer cells bearing multiple genetic lesions) and Reticuloblastoma or RB; genes that specify apoptosis or c ll death such as Fas (Itoh et al, Cell **66**, 233-243, 1991. The polypeptide encod d by the cDNA for human cell surface antigen Fas can mediate apoptosis). GAX (PCT/US95/01882, filed 22.02.95);,and FADD, (Chinnalyan et al. Cell,

81, 505-512, 1995. FADD, a novel death domain-containing protein, Interacts with the death domain of Fas and initiates apoptosis); cell cycle blockers such as GATA-6 (Suzuki et al, Genomics 38, 283-290, 1996. The human GATA-6 gene : Chromosomal location, and regulation of expression by tissue-specific and mitogen-responsive signals); anti-angiogenesis genes such as endostatin and angistatin (Folkman J., Nature Med. 1, 27-31, 1995. Angiogenesis in cancer, vascular, rheumatoid and other diseases.), anti-sense gene sequences (Wang & Becker, Nature Med. 3, 887-893, 1997. Anti-sense targeting of basic fibroblast growth factor and fibroblast growth factor receptor), and genes encoding viral subunit vaccines (Donnelly et al. Nature Med. 1, 583-587, 1995. Pre-clinical efficacy of a prototype DNA vaccine : Enhanced protection against antigenic drift in influenza virus).

Successful approaches to gene therapy require more than the identification and availability of therapeutic genes. The two other key requisites are effective targeting of expression constructs in the body, e.g. in the case of solid tumors to the cancer cells themselves, and finally efficient and effective control of gene expression, i.e. control of the levels and dosage of expressed. targeted therapeutic proteins.

Targeting remains a basic constraint to the effective application of gene therapy, and this particularly when the gene product specified can produce unwanted side effects. This is particularly the case for certain drug mediated cell killing procedures such as the introduction of the herpes thymidine kinase gene (TK) into cells followed by treatment with ganciclovir, which initiates cell death in TK containing cells.

Present approaches to the targeting of therapeutic gene constructs include the use of modified viruses that have some cellular specificity for infection, various anionic and cationic lipids, as well as other non-viral carriers that may have some targeting mechanism such as particle size, lipid solubility or accompanying targeting molecules attached. So far, all of these approaches have met with problems of limited specificity and efficacy.

Other approaches have relied on the use of tissue specific promoters, with the expectation that genes placed under such expression control will be preferentially expressed in the tissue of origin of the tissue specific promoter. Certain limitations appear to be associat d with this approach to date, with for example many tissue specific promoters being either not entirely specific to one cell or tissue type, a lack of

strong tissue specific promoters and a continuing lack of the ability to control expression of genes once targeted.

Amongst the gene switch technologies under development, a need is apparent for a general, easily controlled gene switch that can be employed for multiple purposes and applications.

The present invention in one aspect involves the inducible and controlled expression of a gene, or genes, that can have a therapeutic effect in the treatment of a disease by gene therapy. We and others have explored the applicability of stress promoter-based inducible gene expression over constitutive gene expression in a number of areas such as therapeutic protein production in vitro (US patent 5,646,010 granted July 8, 1997)and *in vivo* (Dreano et al., Biotechnology, November 1988, 1340-1343. Antibody formation against heat-induced gene products expressed in animals.) and in toxicity assessment *in vitro* (Fischbach et al., Cell Biology and Toxicology, 9, 177-188, 1993. Induction of the human growth hormone gene placed under human hsp-70 promoter control in mouse cells: A quantitative indicator of metal toxicity). Further we have previously demonstrated that a marker gene, human growth hormone (hGH) under human hsp-70 expression control, previously integrated into a mouse cell together with a ras oncogene, subsequently injected into nude mice, could be inducibly expressed when the mice were subjected to a whole body hyperthermia (Dreano et al., Biotechnology, November 1988, 1340-1343. Antibody formation against heat-induced gene products expressed in animals.).

Summary of the Invention

In this invention demonstrate that multiple genes can be combined in a specific treatment, and that localized induction can be obtained by external or internal application of a heat source at the site of selected gene activation and protein synthesis. This new development brings the practicability of application of targeted gene expression to gene therapy procedures and this in the absence of high degrees of targeting of the therapeutic gene constructs. Further the induction of therapeutic protein synthesis can be repeated as often as necessary in the selected treatment protocol. This invention thus greatly diminishes the dangerous side effects associated with the use of genes specifying toxic or certain other proteins in gene therapy procedures using non-inducible and non-site directable synth sis of such proteins.

The invention involves the application of stress inducible promoters such as, but not limited to, the human hsp-70 promoter, to direct th controlled expression of one or more therapeutic genes in the body. Using this invention, gene expression can be performed either externally to the body, using sources of controlled heat, or by the insertion of elements enabling local heating of parts of the body. The remarkable finding is that DNA constructs located elsewhere in the body remain inactive and only the successfully heat targeted molecules are activated. This invention thus largely avoids current problems of targeting, particularly of potentially toxic gene expression systems, and further permits a temporal and efficacious control of the delivered expression constructs.

Stress promoters, of which the family of heat shock promoters are probably the best known members, are in general highly controlled promoters. In other words, their non-induced expression is low or non-existent, while their induced levels of expression are very high. Although some of these stress promoters can be activated by multiple stressors such as heat, metal ions, irradiation, and toxic chemicals, the preferred methodology of induction of the expression of therapeutic genes in the invention is that of gentle local heating.

In the field of cancer therapy for instance, there has been considerable hope and effort invested in the use of hyperthermia to treat solid tumors. This approach is based on the apparent greater sensitivity of cancer cells to heat than normal cells. After more than ten years of extensive testing of this approach the results have been somewhat disappointing. More recent observations suggest that higher temperatures than generally employed, even as high as 45°C or even higher, are needed to obtain a differential effect on tumor cells over normal cells.

Irrespective of the limited success of the hyperthermia approach in itself, these developments have resulted in a very considerable development of instrumentation and technologies allowing the controlled heating of specific parts of the body. Instrumentation systems employing microwaves, NMR and other radiation sources have been employed to heat specific parts of the body. One example of such a development is the treatment of benign prostatic hyperplasia (BPH) by a microwave thermotherapy device (the "Prostatron" (R)) distributed by EDAP Technomed Inc, Lyon, France. Such a device can be employed in this invention to control the expression of genes to treat cancer of the prostate, thus opening up a completely new therapeutic field. This is one example of the possible applications of this invention;

other preferred embodiments of the invention will be detailed together with specific examples.

The invention also embraces combined therapeutic approaches in which the use of therapeutic genes placed under stress promoter control is combined with more classical cancer treatments such as chemotherapy using for example cis-platinum and for example radiotherapy.

Detailed Description of the Invention

In one aspect, the invention is directed to a method for treating a subject diagnosed as having a condition that can be treated by gene therapy. The method involves delivering sufficient stress promoter-therapeutic gene constructs to provide effective treatment of such conditions. A subject, as used herein, refers to any mammal that may be susceptible to a condition that can be treated by gene therapy. Preferably the subject is a human.

The invention employs the combination of therapeutic genes, an inducible expression system and specific induction procedures.

It is important, in order to understand the applications of this invention, to look at both constitutive and inducible expression systems and their use in gene therapy :

Expression systems in Gene Therapy

Constitutive gene expression systems in gene therapy

In all of the early work in the field of gene therapy, exclusively constitutive and strong promoters were employed. Examples of such promoters include, but are not limited to the promoters for the following genes : hypoxanthine phosphoribosyl transferase (HPTR), adenosine deaminase, pyruvate kinase, and ß-actin and other constitutive promoters. Constitutive viral promoters which function in eukaryotic cells include those obtained from the simian virus, papilloma virus, adenovirus, human immunodeficiency virus (HIV), Rous sarcoma virus, cytomegalovirus (CMV), the long terminal repeats (LTR) of moloney leukemia virus and other retroviruses. The CMV promoter, a powerful constitutive promoter, has been extensively employed in gene therapy protocols.

Inducible gene expression systems in gene therapy

A number of inducible promot rs have been studied more recently and the importance of inducible expression has come to the fore. Inducible promoters include but are not limited to those of the following genes : metallothionein, heat shock, stress responsive, ubiquitin, ecdysone response element, and doxcycline mediated control of the E. coli tetracycline resistance operon in eucaryotic cells.

Tissue specific expression systems in gene therapy

This growing field includes the following : thyroxin-binding globulin protein gene promoter for the liver, leukocyte integrin CD11 promoters for leukocytes, vascular smooth muscle-specific promoter/enhancer, NSE promoter and the PDGF-B promoter, which appears to be specific to spinal cord neurons, and the rhodopsin promoter which expresses predominantly in photoreceptor cells.

Cell Cycle dependent gene expression in gene therapy

The promoter of the E2F-1 gene controls the expression of a ubiquitous growth regulated protein which exhibits peak activity in S phase. It has been reported that the E2F-1 promoter can drive the expression of transgenes with a specificity of action in dividing cells such as tumor cells (Parr et al., Nature Medicine 3, 1145-1149, 1997. Tumor-selective transgene expression *in vivo* mediated by an E2F-responsive adenoviral vector).

Stress Promoter gene expression in Gene Therapy

The stress promoter driven gene expression approach to gene therapy of this invention can be optionally combined with promoter/enhancer sequences bringing tissue specific and or cell cycle specific expression.

A particularly preferred stress promoter of this invention is the human hsp-70 heat shock gene promoter. Other preferred promoters are the other heat, shock, ubiquitin and stress promoters of human and other eucaryotic origins. Procaryotic stress inducible promoters can also be employed in the invention. Standard protocols for identifying and isolating stress inducible promoter sequences, employing functional assays or the use of reporter genes. Many such inducible promoters have been identified from a wide variety of origins and can be used where appropriate in the invention.

In addition to the isolation of naturally occurring stress promoters, the same or sequence variants of such promoters can be obtained by nucleotide synthesis

procedures and random or site directed mutagenesis, techniques well known to those of ordinary skill in the art (see, for example, Synthesis and Application of DNA and RNA, S.A. Narang, ed., 1987, Academic Press, San Diego, CA and Molecular Cloning, A Laboratory Manual, ed., J. Sambrook et al., Cold Spring Harbor Laboratory Press, Plainview, NY (1989). All such synthetic and sequence variant stress promoters that are stress inducible can be employed in the invention.

Therapeutic gene sequences and stress promoters are said to be "operably linked" when they are covalently linked in such a way as to place the transcription and /or translation of the therapeutic gene sequence under the influence or control of the stress promoter. If it is desired that the inducible therapeutic gene be translated into a functional protein, two DNA sequences are said to be operably linked if induction of a stress promoter in the 5' gene expression sequence results in the transcription of the therapeutic gene sequence, and if the nature of the linkage between the two DNA sequences does not result in the introduction of a frame-shift mutation, does not interfere with the ability of the promoter region to direct transcription of the therapeutic gene sequence, and does not interfere with the ability of the corresponding RNA transcript to be translated into a protein. Further sequences such as 3' termination sequences may be employed where they are necessary to ensure efficient translation of the gene so induced.

The technology of this invention can be applied to a large number of disease states. Preferred applications are those in which diseased tissue is localized in one or more parts of the body. The combination of the optimal choice of therapeutic genes, each placed under stress promoter expression control, and with their expression being directed by external to the tissue stress, and this in a preferred form as gentle heat, are the basis of this invention.

Of the many diseases to which this technology is applicable, solid cancers form an important but not a limiting class of diseases of choice for treatment by this invention. There is essentially no limit to the choice of genes that can be employed, and the specific selection will depend on the particularities of each disease state.

Cancer Gene Therapy

A number of genes are in clinical trials at this time in cancer gene therapy protocols. The types of cancer treatment genes that are available to the medical research community to dat fall into essentially three classes : genes that induce the death of

cancer cells, those that revert the cancer genotype to a non-cancerous form, and those that inhibit the spread of a tumor and the risk of formation of metastases. The invention is applicable to genes from all three classes and in a most preferred embodiment, to the combination of genes from more than one class. In certain preferred embodiments, a specific combination of genes can be combined with standard anti-cancer treatments using chemotherapy, radiotherapy or both.

Genes that can be employed in the invention can include, but are not limited to, one or more of the following :

Apoptosis-inducing genes :

1.1 Genes for proteins with a death domain, e.g. GAX, GATA-6, TNFR1, TRADD, FADD, FAS.

Other approaches have relied on the use of tissue specific promoters, with Th and RIP.

1.2 TRAF family of genes, e.g. TRAF1,TRAF2,TRAF3 and TRAF4/CART1.

1.3 ICE/CED-3 family of genes, e.g. ICE,Ich-1/Nedd-2, Cpp32/Yama/apopain, TX/Ich-2/ICE rel 11, Mch-2, ICE rel 111 and Ced-3

Cell cycle inhibitor genes :

e.g. GATA-6, cdc2 kinase and PCNA,

"Suicide" genes :

e.g. Herpes thymidine kinase gene and ganciclovir or penciclvir, murine alpha-1,3 galactosyl transferase and human serum.

Tumor suppresser genes :

e.g. P 53, NB, p16INK4/CDNK2,

Anti-angiogenesis coding genes :

e.g. Angiostatin, Endostatin,

Anti-sense oligo-nucleotides

Any gene sequence can be employed in its anti-sense mode to block gene expression; target sequences include oncogenes, growth factor genes and angiogenesis inducing factor genes.

The strategic use of a combination of genes in the treatment of specific cancers

This invention has, as one of its preferred embodiments, the treatment of solid tumors, such as those of the brain, the lung and the prostate for example. The procedure adopted is to chose specific genes from the groups described above, and to combine their targeted expression. Since the objective in treating a solid tumor is to prevent its continued growth and expansion, to prevent the formation of metastases, and to eventually eliminate the tumor mass, an astute barrage of induced genes appears optimal.

A general set of genes to treat solid tumors would include one or more members from one or more of the six groups of genes mentioned above. Treatment of prostatic cancers could employ extremely potent genes such as Fas Ligand and tk with gancyclovir, these could be associated with one or more of the growing list of genes that block angiogenesis. Treatment of a brain glioma on the other hand may require a greater reliance of anti angiogenesis genes and cell cycle inhibitor genes, and this to avoid damage to healthy neighboring tissues in the brain. Although the invention indicates the advantage of grouping genes for gene therapy using the given approach, we expect to find precise mixtures of genes with optimal activity in the clinic for individual indications and for specific patient indications. A person skilled In the art could be expected to chose specific mixtures of genes for individual applications based on analysis of pre-clinical results.

Indication other than cancer

Other diseases due to cell proliferation :

A number of vascular disorders result from the proliferation of vascular smooth muscle cells (VSMC) in response to injury. Atherosclerotic lesion formation involves macrophage and T cell infiltration of the vessel wall, inducing VSMC migration from the media to the intima, where these cells dedifferentiate, proliferate, and synthesize extracellular matrix components. These lesions can induce thrombus leading to occlusion of the lumen and distal tissue ischemia. VMSC hyperplasia also contributes to the restonoic occlusion that occurs in 30 – 50% of patients who undergo percutaneous balloon angioplasty. Accordingly a number of groups have explored molecular genetic approaches that target VSMC proliferation to minimize the incidence of restenosis following percutaneous revascularization procedures.

A number of genes have been used, exclusively with constitutive promoters; examples are the thymidine kinase gene (with gancyclovir treatment), Rb, p21 and p27, GAX and Hirudin (Prospects for Intravascular Gene Therapy, Smith R. and Walsh K., Journal of Clinical Apheresis **12**, p. 140-145, 1997). Recent work by Walsh et al., (Sata et al., 1998, Proc. Natl. Acad. Sci. USA **95**, 1213-1217), have more recently employed the Fas ligand gene in such studies. It is probable that with the high level of toxicity of such genes, inducible gene expression will be necessary for clinical applications.

The treatment of diabetes by gene therapy :

This invention, with its precise physical and temporal control of gene expression, in a preferred embodiment outside of the control of diseases due to cellular proliferation, is well adapted to the treatment of diseases that require controlled and dose-related protein production in the body.

One important disease that has these precise characteristics and requirements is diabetes. One important for of diabetes is Insulin-dependent diabetes mellitus; this type of diabetes usually occurs between the ages of 10 and 20 years. The pathogenesis is uncertain but it is probable that in an immunologically susceptible individual, a viral infection of the Beta-pancreatic islet cells leads to self-perpetuating auto-immune damage. While the acute phase of the disease may be followed by a period of partial remission, a virtually complete destruction of the islet cells follows, and a life long requirement for insulin is established.

This invention, applied to the expression of the human insulin gene, would provide a gene therapy solution to the treatment of the disease. Insulin must be provided to patients suffering from is Insulin-dependent diabetes mellitus at specific times and in specific quantities. This invention would allow for instance an ex-vivo approach where encapsulated cells would be engineered to produce controlled levels of insulin, as required.

A number of other diseases also require dosed expression of genes in order to treat the metabolic requirements that are necessary. A number of diseases of the brain and nervous system are best treated by the use of this invention, with temporal and localized expression of specific proteins.

Other diseases such as crises of gout, where there is a temporal lack of specific enzymes involved in uric acid metabolism are also amenable to treatment by this invention.

Metabolic dis ases that require the constant, lifelong production of one or more proteins in the body, such as hemophilia, are not advantageously treated by this invention.

Preferred embodiments of the invention :

The present invention demonstrates that one or more human genes, capable of acting on disease states in subject, and particularly solid tumors, each bringing specific advantages to a treatment. Induction of the expression of such genes in the invention can be performed at specific body sites, by directed stress, preferably gentle heat

The invention can also be employed in a systemic mode in the body using specific pharmaceuticals as inducers for disease treatment.

The invention may be used in an advantageous manner for the expression of genes intended for vaccination purposes. Here a temporal control of expression is desirable, while a physical location of expression is of relatively little importance. An exception where both aspects of control would be required in a DNA vaccine protocol would be where implanted cells are programmed to produce the protein antigen selected for a specific vaccination treatment.

Unfortunately, diseases do not always occur as individual and unique events in human subjects. Older age in particular tends to increase the incidence of a variety of diseases, in particular cancer and cardiovascular disease. In a certain number of cases, a treatment that could be beneficial for the treatment of a cancer, such as the use of anti-angiogenesis inducing compounds or genes, could have an opposite and negative effect on the patient suffering from for instance an ischaemic limb.

Isner et al., (Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patient with ischaemic limb. 1996, J. Isner et al., The Lancet, Vol. 348, p. 370-374), have demonstrated that plasmid DNA encoding phVEGF165 delivered intra-arterially into a patient suffering from an ischaemic right leg showed after four weeks an increase in collateral vessels at the knee, mid-tibia and ankle levels, which persisted at a 12 week view.

These authors employed a single delivery of 2 mg of plasmid DNA in these clinical trials; this considerable amount of DNA delivered into the blood stream for a desired treatment, and the fact that a constitutive promoter, the cytomegalovirus

promoter/enhancer element was employed to express phVEGF165, ensures that other parts of the body will also be affected. Clearly a cancer indication in such patients would risk aggravation by the initial necessary treatment.

This invention permits the expression of two or more genes both in a single location, such as a solid tumor or an artery undergoing restenosis, but also at two or more locations such as the example described above. Thus using one preferred embodiment of the invention, for example an anti-angiogenesis gene such as angiostatin to treat a solid tumor, and an angiogenesis inducing gene such as phVEGF165 to treat ischaemia, both conditions can be advantageously treated while avoiding negative effects on each of the disease states.

Vector and delivery systems for stress promoter-therapeutic gene constructs :

Any of the genes, or combination of genes, given on the non-exclusive list above, can be delivered by a large variety of methods and procedures. They can equally well be introduced into patients cells for *ex vivo* gene therapy as well as for *in vivo* gene therapy applications. Therapeutic genes placed under stress promoter expression control can be delivered to cells or tissues alone or in association with a vector. In the broadest sense, a "vector" is any vehicle capable of facilitating delivery of the gene expression system to a target cell, as well as uptake of the expression system into the target cell with less degradation than would occur in the absence of a vector.

Vectors can be divided into essentially two classes; biological vectors and chemical/physical vectors. Biological vectors include but are not limited to plasmids, phages, cosmids, viruses, vehicles derived from bacterial or viral sources into which the nucleic acid sequences of the invention can be inserted or incorporated. Viral vectors are a preferred type of biological vector and include, but are not limited to the following viruses : adenovirus, adeno-associated virus, herpes virus, vaccinia virus, polyoma and papilloma viruses, semliki forest virus, retroviruses such as rouse sarcoma virus, harvey murine leukemia virus, moloney murine leukaemia virus, human immunodifficiency virus. Other virus vectors not named but known in the art may also be employed.

Preferred viral vectors are those that infect eucaryotic cells without cytopathic effects and which have been modified to replace non-essential or potentially dangerous genes with other genes including the therap utic gene or genes plac d under stress promoter expression control. Adenovirus-based and retrovirus- based vectors as well as recently

described adenoviral/retroviral chimeric vectors (Feng et al.., Nature Biotechnology Vol 15, September 1997. Stable *in vivo* gene transduction via a novel adenoviral/retroviral Chimeric Vector) are preferred viral vectors for the invention.

Preferred non-viral vectors include colloidal dispersion systems which include lipid-based systems including oil in water emulsions, micelles and liposomes. Liposomes may be targeted to particular tissues by coupling the liposome to a specific ligand, such as a monoclonal antibody, sugar, glycolipid, or protein. Additionally, the vector may be coupled to a nuclear targeting peptide, which could direct the stress promoter-therapeutic gene constructs to the nucleus of the host cell.

In one particular embodiment, the preferred vehicle is a bio-compatible micro sphere or implant that can be introduced into the body. Exemplary biodegradable implants that can be employed with this invention are described in PCT international application no. PCT/US/03307. This system describes a bio-compatible, biodegradable polymeric matrix for containing exogenous genes under suitable promoter expression control. The polymeric matrix is employed to achieve sustained release of the exogenous gene in the patient. Other forms of polymeric matrices, including films, coatings, and gels can also be employed.

In another preferred embodiment, the preferred vehicle is a cell of mammalian origin that contains the stress promoter-therapeutic gene constructs selected for a particular disease application. Viable cells carrying the expression constructs of the invention, in, for example, 3-dimensional particulate, non-crosslinked chitosan core matrices, as described in patent application WO 93US9023, can be encapsulated as therapeutic implant devices, prepared by co-extruding viable cell cultures and polymeric solutions, and sealing such tubular semi-permeable extrudates; see patent application US 5283187. Such implants can be used as *in vivo* bio-reactors for the production of useful proteins in the body, using the technology of this invention.

In the therapeutic applications of the invention the therapeutic molecules are administered in effective amounts. In general, a therapeutically effective amount means the amount necessary to delay the onset of, inhibit the progression of, or halt altogether the particular condition being treated. Generally this amount will vary with the age, sex and weight of the patient, as well as the natur and stage of the disease being treated, all of which can be determined by one of ordinary skill in the art.

Gene therapy using stress promoter-therapeutic gene constructs can be performed either alon or in combination with conventional drugs and other therapi s. For instance, in the case of cancer treatment, such as cis-platinum, Vinblastine, Taxol, Vepeside, Caryolysine, bleomycine amongst other chemotherepeutic drugs, and/or radiotherapy, may be combined with treatment using the invention.

Others have developed vectors and system approaches for the delivery of genes and proteins for gene therapy applications. examples of such developments are quoted here for reference and in order to clearly specify their differences from this invention :

1. Vectors and vector systems including genes encoding tumor suppresser proteins and producer cells transformed thereby, PCT/US94/14278.

Here, an inducible promoter is employed, and a series of inducible promoters are cited, including heat shock promoters. The objective and examples of this technology are however different from those of the invention. Their objective is to control the level of a tumor suppresser protein production in producer cells, allowing the cells to proliferate and to generate recombinant viral particles. These particles are then employed in gene therapy. The inducibility is employed to control the level of tumor suppresser protein produced; further all examples given describe producer cell lines for the viral vectors as well as the vectors themselves.

2. Pore-forming and super-antigen encoding toxin cassettes for Gene Therapy, PCT/US94/07091.

Here, the genes chosen for gene therapy applications encode toxin genes, and preferably genes encoding cytolytic enterotoxins which disrupt the plasma membrane integrity by generating pore-forming complexes. An example of such a toxin is the Staphylococcus aureus enterotoxin A (SAEA). Both heat shock and ionizing irradiation inducible promoters are proposed as controlled expression elements for SAEA. This demonstration and claimed applications are strictly limited to genes specifying potent toxins, genes of non-human origin.

The demonstration of the use of inducible promoters, such as the human hsp-70 promoter, to express gen s in animal tumors, has be n reported (Dr ano et al., Biotechnology, November 1988, 1340-1343) Further, the demonstration of the control of c-myc gene expression by the Drosophila melanogaster hsp-70 promoter in mouse

cells (Wurm et al., Proc. Natl. Acad. Sci. US, **83**, 5414-5418, 1986) is an elegant example of the extremely tight expression control of the hsp-70 promoter utilized. When these cells were induced by a heat shock, a very significant quantity of c-myc was synthesized, leading to cell death.

Experimental protocols

Example 1: Construction of the expression plasmid Hhsp-70-vector

The base plasmid employed was the commercially available pBK-CMV phagemid of 4518 base pairs (Stratagene, GmbH, Postfach 10 54 66, D-69044 Heidelberg 1, Germany). This plasmid permits both procaryotic replication and propagation, as well as eucaryotic expression. In eucaryotic cells expression is controlled by the CMV immediate early promoter. The objective is to replace the constitutive CMV promoter with the human hsp-70 inducible promoter.

pBK-CMV was digested by a mixture of Hind 111 and Apa L1. The larger fragment, 3,329 base pairs was isolated. pBK-CMV was digested with a mixture of Apa L1 and Nsi 1 and the resulting 363 base pair fragment was purified. The other fragment sizes are 1,962, 70 and 2,030 base pairs respectively. Plasmid p17 (Voellmy et al., P.N.A.S. USA, **82**, 4949-4953, 1985. Isolation and functional analysis of a human 70,000 dalton heat-shock protein gene segment.) was digested with a mixture of Pst 1 and Hind 111 and the 700 base pair fragment was isolated. It should be noted that Pst 1 and Nsi 1 are iso-acceptors.

The three isolated fragments described above, respectively 3,329, 363 and **700** base pairs were mixed together and ligated; the ligation mixture was used to transform E. coli and selected for kanamycin resistance. The resulting plasmid, Hhsp-70 was purified and characterized using standard procedures.

Example 2: Construction of the expression plasmid Hhsp-70-GATA-6

Hhsp-70 was digested with a mixture of Xba 1 and Apa 1 and the larger fragment of 3,311 base pairs was isolated; the smaller fragment is of 1,800 base pairs. In a separate digest, Hhsp-70 was digested with a mixture of Hind 111 and Apa L1 and the smaller fragment of 1,800 base pairs was isolated; the larger fragment is of 3,329 base pairs. The GATA-6 containing plasmid pCGN (Suzuki et al., Genomics, **38:3**, 283-290, 1996. The human GATA-6 gene : structure, chromosomal location, and regulation of

expression by tissue-specific and mitogen-responsive signals) was digested with a mixture of Hind 111 and Xba 1, and th GATA-6 containing fragment of 1995 base pairs was isolated. Hhsp-70-GATA-6 was prepared by ligating the three fragments described above and transforming E. coli using kanamycin selection.

Example 3: Construction of the expression plasmid Hhsp-70-p53

Plasmid Hhsp-70-p53 was constructed by substituting the GATA-6 sequences present in plasmid Hhsp-70-GATA-6 with the p53 sequences present in a wild type p53 cDNA-containing plasmid. This was done in the following way : Plasmid Hhsp-70-GATA-6 was digested with a mixture of Hind 111 and Xba 1, and the larger resulting fragment of about 5,200 base pairs was purified; the smaller fragment is of 1995 base pairs. In a separate reaction mixture, the wild type p53 sequences present in SN3-p53 were amplified by PCR through use of oligo-nucleotides of the following sequence :

5' TTAATTAATTAAGCTTATGGAGGAGCCGCAGTCAG 3'

containing a Hind 111 site (underlined), and

5' TACTGATATAAATCTAGACTAGTCTGAGTCAGGCCCTTCTG 3'

containing an Xba 1 site (underlined).

These oligo-nucleotides were chosen to add unique Hind 111 and Xba 1 sites at the 5'- and 3'- ends respectively of the p53 gene. The amplified PCR product was digested with a mixture of the Hind 111 and Xba 1 endonucleases, and the resulting fragment of around 1,180 base pairs was isolated and ligated to the 5,200 base pair fragment described above, yielding plasmid Hhsp-70-p53.

Example 4: Construction of a replication-defective adenovirus expressing the GATA-6 gene under hsp promoter control

Adenoviral expression vectors were produced by the method developed by M. Perricaudet and his group, (Smith R.C., Branellec D., Gorski D.H., Guo K., Perlman H., Dedieu J.-F., Pastore C., Mahfoudi A., Denèfle P., Isner J.M. and Walsh K. 1997, p21 CIPI-mediated inhibition of cell proliferation by over-expression of the *gax* homeodomain gene. Genes Dev. **11**: 1674-1689). Plasmid pBK-HSP-GATA-6 was digested with a mixtur of Bam H1 and Xba 1 restriction enzymes, and the smaller resulting fragment of about 2,000 bp containing the HSP and GATA-6 sequences was

made flush ended by treatment with the Klenow fragment of DNA polymerase in the presence of the four deoxyribonucleoside triphosphates.

In parallel the pCO1 vector described in the above reference which contains the adenovirus 5 sequences required for homologous recombination was linearized by digestion with Eco RV and was dephosphorylated by treatment with the calf intestine alcaline phosphatase (CIP). It was then ligated to the HSP-GATA-6 containing fragment. After transformation into competent E coli cells, pCO1 derivatives were obtained, harboring the HSP-GATA-6 gene in either of two orientations. Such plasmids were linearized with the Xmn1 endonuclease and were co-transfected into 293 cells together with the large Cla 1 fragment of the Adeno 5 d1234 viral DNA (Stratford-Perricaudet L.D., Makeh I., Perricaudet M. and Briand P. 1992, Widespread long-term gene transfer to mouse skeletal muscles and heart. J. Clin. Invest. **90**, 626-630). In this method, infectious viral particles expressing GATA-6 under HSP control are generated by recombination in the 293 cells.

All of these described plasmids were derived from serotype 5 human adenovirus from which the viral early genes E1a, E1b and E3 were deleted. The resulting adenovirus is limited to propagation in 293 cells which produce the Ad5 E1 gene products required for replication. The recombinant, replication-defective adenoviruses are purified from isolated plaques and viral DNA is prepared. Recombinant adenovirus containing the appropriate expression cassettes can be identified by restriction fragment analysis and amplified in 293 cells. The viral preparations are purified by two CsCl gradient centrifugations. Viral titer is determined by plaque assay on 293 cells (Graham F.L. and van der Eb A.J. 1973, A new technique for assay of infectivity of human adenovirus 5 DNA. Virology **52**, 456-463).

Example 5: Construction of adenoviral vectors expressing the full-length native Human Fas ligand and a derivative form lacking amino acids 430-460, spanning a region of the protein that is permissive for shedding of the extra-cellular domain.

The full length human Fas ligand cDNA (Takahashi, T., Tanaka, M., Inazawa, J., Abe, T., Suda,T. and Nagata, S. Human Fas ligand: gene structure, chromosomal location and species specificity. Int. Immunol. **6**, 1567-1574 (1994)) was obtained in the vector pEX-hFL1 from Shigekazu Nagata (GenBank Accession U11821). Th Fas ligand open reading frame was obtained with flanking Xhol (5') and Xbal (3') restriction sites by PCR amplification utilizing the prim rs :

Kpn5 (5' GGGGGGGGTACCCTCGAGATGCAGCAGCCCTTCAATTACCCA 3')

and

Xba3 (5' GGCGGCTCTAGATTAGAGCTTATATAAGCCGAAAAACG 3').

The translational start and stop signals are underlined. The resulting PCR reaction was size fractionated by agarose gel electrophoresis and the anticipated product (predicted size to be 876 bp) was purified utilizing Qiaex II (Qiagen). The purified fragment was digested with the KpnI and XbaI restriction enzymes (New England Biolabs) and the restriction digest was size fractionated and purified. The eluted fragment was ligated to a 1710 bp KpnI/XbaI digested fragment of the pGL3-Basic vector (Promega) containing the bacterial origin and ampicillin resistance genes but lacking the luciferase reporter gene. The ligation mixture was transformed into XL1Blue competent bacteria and recombinants selected for ampicillin resistance. Individual isolates were screened by restriction enzyme analysis for the presence of the 861 bp Fas ligand XhoI/XbaI fragment. The resulting recombinant plasmid containing the Fas ligand cDNA was digested with XhoI restriction enzyme and the linearized plasmid was size fractionated and purified. The purified plasmid was ligated to an approximately 400 bp XhoI/SalI fragment, derived from a restriction enzyme digest of pD3SX (StressGen Biotechnologies Corp.), containing the Hsp70B promoter and RNA leader sequences. The recombinant plasmid was transformed into bacteria and selected for ampicillin resistance. Plasmids from isolated bacterial colonies were screened for the proper orientation of the Hsp70B promoter by restriction enzyme analysis utilizing the XhoI and XbaI restriction enzymes. Recombinants containing an approximately 1300 bp fragment were chosen (pGL3-Hsp70B Fas ligand) for analysis of induction of the HSP70B promoter and expression of functional Fas ligand.

A derivative form of Fas ligand resistant to shedding was created by deletion of a cleavage site located between amino acids 129 and 130 (Tanaka, M., Itai, T., Adachi, M., Nagata, S. Down regulation of Fas ligand by shedding. Nature Med. 4, 31-36 (1998)). The deletion contained an in-frame fusion of the amino-terminal coding region of Fas ligand (amino acids 1-121) to the carboxyl-terminal coding region (amino acids 133-281). This was accomplish d by separat PCR amplifications of the amino-terminal region from pEX-hFL1 using the Kpn5 primer in combination with the primer

FL5R (5' GGGTGGACTGGGGTGCATCTGGCTGGTAGACTCTC 3')

and the carboxyl-terminal region using the primers :

FL3F (5' GAGAGTCTACCAGCCAGATGCACCCCAGTCCACCC 3')

and Xba3 (see above).

The PCR reactions were size fractionated and the correct size PCR products were purified. The 5' (396 bp) and 3' (477 bp) fragments were mixed in a 1:1 ratio and a full-length template was generated by extension of the cross-primed hybrids made possible by the complementary sequences (underlined) within the FL5R and the FL3F primers. This template was then amplified by PCR utilizing the Kpn5 and Xba3 primers. The PCR reaction was fractionated by agarose gel electrophoresis and an 843 bp product was identified, isolated and purified. The PCR product was then digested with the KpnI and XbaI restriction enzymes and the restriction digest was fractionated, purified and placed within the PGL3 basic vector under control of the HSP70B promoter in the manner described for the full-length native Fas ligand. Those recombinants containing an approximately 1200 bp fragment were chosen (pGL3-Hsp70B ncFas ligand) for analysis of induction of the Hsp70B promoter and the expression of a form of Fas ligand resistant to shedding into the culture medium.

Construction of adenoviral vectors was achieved by standard protocols (Becker, T. C., Noel, R. J., Coats, W. S., Gomez-Foix, A. M., Alam, T., Gerard, R. D., Newgard, C. B. (1994) *Use of Recombinant Adenovirus for Metabolic Engineering of Mammalian Cells* in «Methods in Cell Biology» vol. 43, Chapter 8, pp. 161-189, (Academic Press)). The CMV promoter and SV40 polyadenylation signal sequences were excised from the pACCMV.pLpA plasmid by digestion with the NotI restriction enzyme and size fractionation of the restriction digest by agarose gel electrophoresis. The fragment containing the Ad5 sequences (approximately 7.6 kb) was identified, isolated and purified. KpnI and XbaI sites were created by ligation of the NotI fragment of pACCMV.pLpA to a pair of oligonucleotides NKX1 (5' GGCCGGTACCTCTAGA 3') and NKX2 (5' GGCCTCTAGAGGTACC 3'). A recombinant plasmid containing XbaI and KpnI restrictions sites (pACCMV.pLpA-KX) was identified by screening candidates for the presence of a single fragment upon agarose gel electrophoresis of restriction digests of the plasmid with KpnI or XbaI. The pGL3-Hsp70B Fas ligand construct was digested with the KpnI and XbaI restriction enzymes. The resulting digest was size

fractionated by agarose gel electrophoresis and the Hsp70B promoter fragment (1300 bp) was identified, isolated and purifi d. These fragments were ligated to the 7.6 kb KpnI and XbaI restriction fragment of pACCMV.pLpA-KX containing the Ad5 sequences necessary to obtain recombination with the pJM17 plasmid that contains a modified Ad5 genome. Recombinant constructs were identified by digestion with the KpnI and XbaI restriction enzymes and identified by the presence of a 1300 bp restriction fragment upon analysis by agarose gel electrophoresis. The pACCMV.pLpA Fas ligand construct was co-transfected with pJM17 into the 293 human cell line by $CaCl_2$ precipitation. When the culture reached maximal lysis, the monolayer was detached and virus was obtained by freeze-thaw lysis. The resulting lysate was amplified and serial dilutions were used to infect monolayers of 293 cells that were then cultured under a soft agarose overlay. Plugs containing single plaques were taken from plates infected at high enough dilutions of virus to allow the formation of isolated plaques. The plugs were used to prepare viral stocks to identify positive recombinants expressing Fas ligand. Positive clones were characterized for their ability to express Fas ligand under temperature control of the Hsp70B promoter. An identical process was used to obtain an adenoviral construct from pGL3-Hsp70B ncFas ligand expressing a non-cleavable form of Fas ligand.

Example 6 : *In vitro* testing with GATA-6 expression plasmids.

Mouse embryonic fibroblasts (MEF's) were induced to the quiescent state by serum deprivation for three days in 0.5% FBS DMEM. MEF's were cultured in 60mm plates containing gelatin-coated coverslips at a density of 50,000 cells/ml. After induction of the quiescent state, cells were transfected with Hhsp-70-GATA-6 (example 2 below) in OPTI-MEM media for four hours with 5 micrograms of construct using the LipofectAmine protocol (Gibco BRL). After four hours of incubation, cells were washed twice with PBS at 37 ' C and were subsequently cultures for 12 hours in 10% FBS DMEM. Control transfections were also performed with ß-galactosidase expression plasmids and with GATA-6 expression plasmids, where the GATA-6 gene was placed under the control of the powerful, constitutive promoter from CMV.

For the expression experiments, all transfected cultures were subjected either to a heat shock, 42 'C for 90 minutes, followed by incubation at 37'C for 16 hours. At this point, 10 micro moles of BrdU was added to the media and cultures were incubated for an additional 12 hours. GATA-6 xpression and BrdU incorporation were detected by double immunofluor scenc . R presentativ microscopic fields demonstrated that only GATA-6 positive cells, CMV-GATA-6 plasmids with or without heat shock, and Hhsp-

70-GATA-6 plasmids exclusively after heat shock, did not incorporate BrdU. This demonstrates that the expression of a cell cycle inhibitor gene, such as GATA-6, can be precisely controlled *in vitro* using Hhsp-70 expression control.

Example 7 :*In vivo* testing with Adenoviral-mediated gene therapy of a rat model of restenosis.

Viral preparations used for *in vivo* studies were purified by CsCl gradient centrifugation and dialyzed against buffer containing 10 mM Tris-Cl pH 7.4, 10% glycerol and stored at -80°C. Viral titer was determined by plaque assay on 293 cells and expressed as plaque forming units (pfu) per ml.

The model of balloon injury was based on that described by Clowes *et al.* (Clowes, A.W., M.A. Reidy, and M.M. Clowes. 1983. Kinetics of cellular proliferation after arterial injury I: smooth muscle cell growth in the absence of endothelium. *Lab. Invest.* **49**:327-333). Male Sprague-Dawley rats weighing 400-500g were anesthetized with an intraperitoneal injection of sodium pentobarbital (45 mg/kg). The bifurcation of the left common carotid artery was exposed through a midline incision and the left common, internal, and external carotid arteries were temporarily ligated. A 2F embolectomy catheter was introduced into the external carotid and advanced to the distal ligation of the common carotid. The balloon was inflated with saline and drawn towards the arteriotomy site 3 times to produce a distending, de-endothelializing injury. The catheter was then withdrawn. Adenovirus (1×10^9 pfu in a volume of 10 µl diluted to 100 µl) was injected via a cannula, inserted just proximal to the carotid bifurcation into a temporarily isolated segment of the artery. The adenovirus solution was incubated for 20 min after which the viral infusion was withdrawn and the cannula removed. The proximal external carotid artery was then ligated and blood flow was restored to the common carotid artery by release of the ligatures.

Induction of expression of test genes was performed by local heating of the treated carotid area using a plastic water bath conforming to the carotid, and a temperature of 44'C was maintained for 30 minutes. This procedure was performed at the time of adenoviral treatment and subsequently every three days.

Rats were sacrificed at 14 days following treatment with an intraperitoneal injection of pentobarbital (100 mg/kg). The initially balloon injured segment of the left common carotid artery, from the proximal edge of the omohyoid muscle to the carotid bifurcaton, was perfused with saline and dissected free of the surrounding tissue. The

tissue was fix d in 100% methanol until imbedded in paraffin. Several 4-µm sections were cut from each tissue specimen. One section from each specim n was stained with hematoxylin and eosin and another with Richardson's combination elastic-trichrome stain for conventional light microscopic analysis.

Histological images of cross sections of hematoxylin and eosin or elastic-trichrome stained arterial sections were projected onto a digitizing board and the intimal, medial and luminal areas were measured by quantitative morphometric analysis using a computerized sketching program.

The effect of Fas ligand placed under the expression control of the constitutive CMV promoter on neointimal formation in rat carotid arteries at 2 weeks following balloon injury has been published (Sata et al., 1998). The results indicate that with Fas ligand, increased dosage from 1 x 10^6 to 3 x 10^6 pfu per animal gives comparable results. Over a 300 fold difference in viral concentration gives a relatively similar degree of efficacy, from a 54% to 73% decrease in neointima/intima. This indicates that a threshold effect is observed with Fas ligand. Thus the studies performed with hsp-70 promoters, even though they are inducible and not constitutive, and are somewhat weaker than the very powerful CMV promoter, indicate a similar degree of efficacy. The intrinsic advantage of the stress inducible promoter over CMV in this application is its localized expression in the rat carotid.

Figure 1 indicates the comparative effect of adenoviral constructs expressing Fas ligand under control of the CMV promoter and those under HSP promoter control. The results shown are from arteries 2 weeks after balloon injury and adenoviral treatment; adenoviral treatments utilized 1 x 10^8 pfu/animal. The results indicate an approximately 60% inhibition for both promoters compared to the saline and β - galactosidase samples.

Example 8 : Adenoviral-mediated gene therapy of a rat brain tumor model of glioma.

A rat model of glioma was used to test for efficacy of treatment by adenovirus mediated gene transfer. An undifferentiated glioma cell line, F98, derived from a glioma induced by exposure of a pregnant CDF Fischer rat to 50 mg/kg of N-methyl-N-nitrosourea on the 20th day of gestation was utilized for implantation. Tumor implantation was performed in a modification of the procedure described by Kobayashi et al. (Kobayashi, N., Allen, N., N. R. Clendenon, L. W. Ko. 1980. An improved rat

brain-tumor model. *J Neurosurg.* 53:808-815.) and adapted for F98 implantation by Clendenon et al. (Clendenon, N. R., R. F. Barth, W. A. Gordon, J. H. Goodman, F. Alam, A. E. Staubus, C. P. Boesel, A. J. Yates, M. L. Moeschberger, R. G. Fairchild, and J. A. Kalef-Ezra. 1990. Boron neutron capture therapy of a rat glioma. *Neurosurgery* 26:47-55) and with adenoviral treatment as described by Perez-Cruet et al. (Perez-Cruet M. J., T. W. Trask, S. H. Chen, J. C. Goodman, S. L. Woo, R. G. Grossman, H. D. Shine. 1994. Adenovirus-mediated gene therapy of experimental gliomas. *J. Neurosci. Res.* 39:506-511).

In order to apply the invention to this *in vivo* model, an appropriate Implant probe was constructed and employed as follows; this probe resembles in some ways the Ommaya Reservoir system used in the treatment of a variety of brain cancers (Ommaya A.K. 1984, Implantable devices for chronic access and drug delivery to the central nervous system, Cancer Drug Deliv. **1(2)**, 169-179, and Cornwell C.M. 1990. The Ommaya Reservoir: implications for pediatric oncology. : Pediatr.Nurs. **16(3)** 249-251).

- The probe is constructed from a hard polymer (such as teflon) molding in the general shape of a coach bolt, drilled out through from the wide end, and containing miniaturized resistors for heating and their wiring. The internal volume communicates with the CSF through a large number of radial holes in the stem.
- Several initial probes were also equipped with a temperature sensor placed flush with the outside surface, insulated from the heated volume inside, and at varying depths, to allow the study of heat diffusion in those rats' brains.
- The other, larger end of the implant is hollowed and closed with a self-healing membrane. The wires are allowed out through a radial sealed hole. This membrane is used to allow passage of the Adenoviral material using a standard hypodermic needle.
- A thread is placed at the top of the stem, immediately under the reservoir, allowing the implant to be securely fastened to the rat skull.

Method of insertion

- The probe is inserted into the rat brain through the hole that was used to inject the tumor tissue, using th manipulating arm of a stereotactic frame and gently inserted into the brain to its maximum depth and screwed into place.

Injection of Adenoviral vector suspension

- Before the Adenoviral vector suspension in injected, an equal amount of CSF is slowly removed from the reservoir. The Adenoviral solution is then injected and the heaters are turned on to promote convection of the solution out of the reservoir stem.

Determination of heat diffusion parameters

- Initial experiments on dead rat brains, followed by several live experiments using reservoir stems equipped with temperature sensors allowed the creation of a simple rat brain thermal model, which is then used to create the resistor current-voltage profiles for excitation of the construct in the tumor tissue.

Method of induction

- Heat is applied by applying a DC voltage to the resistors, measuring the current. The resistor temperature is inferred from their thermal voltage-current characteristic, which is calibrated beforehand, and serves to avoid thermal breakdown of the resistors. The resistor temperature profile is controlled in such a way as to produce a temperature not exceeding 44°C within the CSF around the exterior of the reservoir stem.

It is understood that the method of heat induction indicated here is not limiting. Microwave, laser, magnetic induction procedures for example are all appliacable to specific or multiple applications of the invention. In the specific case of stent implantation for restenosis treatment for example, the use of metallic stents permits the use of magnetic induction heating specifically on the stent. This allows the expression of genes such as Fas ligand, GATA-6, GAX and others to be expressed precisely and uniquely at the site of potential cell proliferation in balloon treated arteries.

In this procedure, adult female Fischer rats were anaesthetized by injection with ketamine (42.8 mg/ml), xylazine (8.6 mg/ml) and acepromazine (1.4 mg/ml) and were secured within a stereotaxic frame. A midline incision was made in the scalp and an opening was made with a 0.9 mm drill at a point 1.8 mm right and 2.5 mm anterior to the bregma. A skull embedded plastic screw was used as a guide. Injections of 5 x 10^4 viable tumor cells in 10 ul of Dulbecco's minimal essential media containing 1% agarose was loaded into a syringe fitted with a 26 gauge needle and fixed to the

manipulating arm of a stereotaxic frame. The inj ction was made at a depth of 4.5 mm from the dura into the right caudate nucleus over the course of several minutes. Th needle was left in place after the injection for several minutes and then was withdrawn slowly. The hole in the screw was filled with bone wax and the scalp was closed with sterile clips.

Four to eight days after tumor injection 1.2 x 10^9 adenoviral particles in sterile 10 mM Tris-HCl pH 7.4, 10% glycerol in 6 microliters. Injections were made at the same coordinates used for the implantation of the tumor cells. Injections began at a depth of 5.5 mm below the dural surface and 6 injections of 1 ul as the needle was raised in 0.5 mm increments. Injections were made slowly over the course of several minutes and the needle was withdrawn slowly.

Upon completion of the studies the animals were euthanized by injection of mg/kg of sodium pentobarbital and fixed by cardiac perfusion with phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. The brain was fixed for an additional 24 hours following removal and then prepared for frozen sections by successive incubations in fixative containing 5%, 10% and 20% sucrose and embedded in OCT. Cryostat sections (6 micron) were stained with hematoxylin and eosin for light microscopic analysis. Therapeutic response was measured as the survival time in days at 4 days following treatment with adenovirus. The results obtained are as follows :

Example 9 : F98 model of brain glioma employing multiple gene therapy :

Untreated animals	Survival time between 20 to 30 days
HSP-Fas ligand treated	50% alive after 27 days 100% mortality after 36 days
HSP-Fas and HSP-GATA-6	50% mortality after 30 days
HSP-Fas and HSP-GATA-6	100% mortality after 40 days

The results indicate that the use of an individual gene under the indications given, and using a powerful and potentially dangerous gene such as Fas ligand, has a life increasing effect in this animal model. The combination of genes under the terms of this invention increases this protective effect.

It is to be noted that the objective of the application of multiple gen s under strict temporal and targetable xpression control is not simply to obtained an increase in cell killing in tissue related to cell proliferation diseases such as cancer and restenosis. The clinical effect sought involves both efficacy and the absence of secondary effects due to any potential leakage of gene expression in tissues other than the targeted tissue.

Clearly a man skilled in the art would be able to test multiple combinations and dosages of therapeutic genes in any particular indication, be this cancer or other diseases. The studies reported are a demonstration of the general principle of the invention.

Legend to Figure 1

Adult Fischer rats were treated as described in the text to example 7. The standard viral doseage in the rat model is 1 x ao^9 pfu total, with either asingle gene vector or with the employed combination as described in Example 8. The effects on neointimal formation in the balloon injury model of the rat carotid artery are shown in the graphic to figure 1. From left to right:

- An 'empty' adenoviral vestor.
- The Fas ligand adenoviral vector employing the human HSP-70 promoter
- The Fas Ligand adenoviral vector employing the CMV constitutive promoter.
- An adenoviral vector containing the β-galactosidase gene under CMV promoter expressio control.

The procedure employed for these analyses is described in the text to Example 7.

The foregoing description has been directed to particular embodiments of the invention in accordance with the requirements of the Patent Statutes for the purposes of illustration and explanation. It will be apparent, however, to those skilled in the art that many modifications and changes will be possible without departing from the spirit and scope of the invention. It is intended that the following claims be interpreted to embrace all such modifications and changes.

CLAIMS

1. A method for treating a subject diagnosed as having a disease that can be treated by gene therapy, comprising :
   - cleaving DNA having a eucaryotic heat inducible gene to produce a promoter sequence of said gene and excluding the structural gene sequence coding for the heat shock protein;
   - preparing recombinant transfer plasmids by combining a suitable transfer plasmid with said promoter sequence as a first step and secondly with DNA sequences encoding for selected heterologous mixtures of proteins, wherein the selected heterologous DNA sequences are operably linked to the heat shock promoter, such that they are under the transcriptional and translational control of said promoter, providing expression constructs;
   - administering said expression constructs to the subject, in an amount effective to prevent or reduce a disease state;
   - inducing the expression of the selected heterologous genes by a stress treatment of the part of the body to be treated, sufficient to induce expression of the heat shock promoters therein;

2. The method of claim 1, further comprising the induction of heat shock promoters by sources of electromagnetic, ionizing, infra red, ultra violet, microwave, heat, laser, ultrasound and nuclear magnetic resonance emissions.

3. The method of claim 1, further comprising the induction of heat shock promoters by stress inducers such as metal salts, other organic or inorganic compounds, pharmaceuticals, hormones and chemotherapeutic drugs.

4. The method of any preceding claim whereby anti-sense gene sequences are employed in place of DNA sequences encoding for selected heterologous proteins.

5. Th m thod of any claim from 1 to 3 whereby th therapeutic g ne- expr ssion constructs combin genes encoding at least two proteins

s lected from those that induce apoptosis, that suppress tumors, and that inhibit angiogenesis.

6. The method of claims 1-3 whereby the therapeutic gene-expression constructs are selected for the treatment of genetic diseases other than cancer.

7. The method of any preceding claim wherein the gene therapy treatment includes the administration of standard cancer therapy protocols, selected from the group consisting of chemotherapy, radiotherapy and immunotherapy.

8. The method of any preceding claim whereby the therapeutic gene-expression constructs are introduced into the subject as naked DNA.

9. The method of claims 1 - 7 whereby the therapeutic gene-expression constructs are introduced into the subject contained in or associated with a vector.

10. The method of claim 9 a chemical/physical vector is employed.

11. The method of any preceding claim whereby the therapeutic gene-expression constructs are introduced into the subject contained in or associated with slow release formulations.

12. The method of claim 9 whereby the therapeutic gene-expression constructs are introduced into the subject contained in or associated with viral vectors.

13. The method of any preceding claim whereby the therapeutic gene-expression constructs are introduced into the subject for the specific treatment of cancer.

14. A composition comprising : a gene sequence specifying an anti-angiogenesis-inducing protein such as Angiostatin or endostatin, placed under a heat inducible promoter expression control, that permits expression of said gene *in vivo*; and a vector associated with said gene construct.

15. A composition comprising : a gene sequence specifying a tumor suppressor protein such as P53, p16INK4/CDNK2.

16. A composition comprising : a g n sequence specifying an apoptosis-inducing protein such as GAX, TNFR1, TRADD, FADD, TRAF1, TRAF2, TRAF3 and TRAF4/CART1, ICE, Ich-1/Nedd-2, Cpp32/Yama/apopain,

TX/Ich-2/ICE rel 11, Mch-2, ICE rel 111 and Ced-3, Bcl-xS, Bax, Bad, Bak and Bik.

17. A composition comprising : a gene sequence specifying an anti-sense sequence.

18. Compositions comprising : gene sequences specifying proteins selected from the groups of apoptosis inducing proteins, tumor suppresser proteins, cell cycle inhibitor proteins, angiogenesis-inducing proteins and anti-sense sequences.

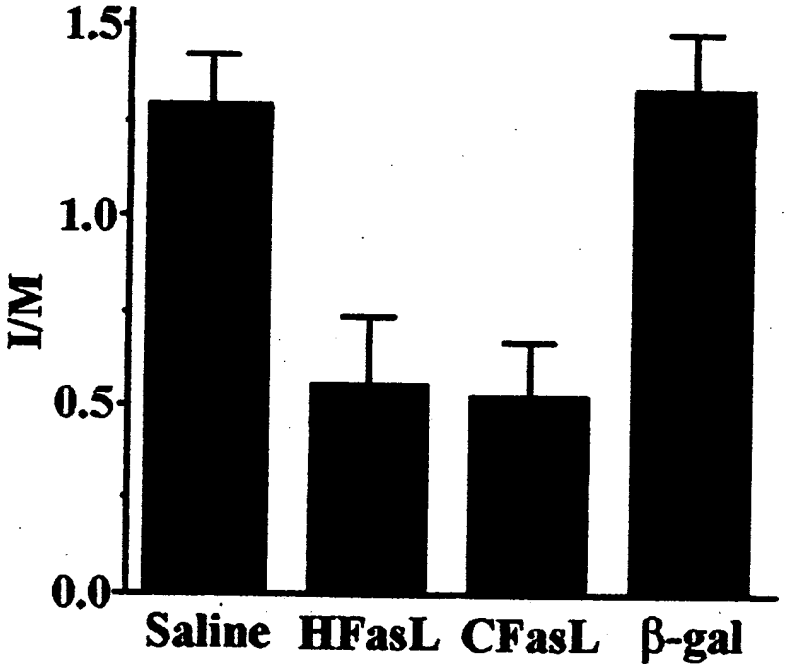

FIG. 1